



## The *in vitro* redundant enzymes PurN and PurT are both essential for systemic infection of mice in *Salmonella enterica* serovar Typhimurium

Jelsbak, Lotte; Mortensen, Mie Ina Bjerregaard; Kilstrup, Mogens; Olsen, John E.

*Published in:*  
Infection and Immunity

*Link to article, DOI:*  
[10.1128/IAI.00182-16](https://doi.org/10.1128/IAI.00182-16)

*Publication date:*  
2016

*Document Version*  
Peer reviewed version

[Link back to DTU Orbit](#)

*Citation (APA):*  
Jelsbak, L., Mortensen, M. I. B., Kilstrup, M., & Olsen, J. E. (2016). The *in vitro* redundant enzymes PurN and PurT are both essential for systemic infection of mice in *Salmonella enterica* serovar Typhimurium. *Infection and Immunity*, 84(7), 2076-2085. <https://doi.org/10.1128/IAI.00182-16>

---

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1  
 2 The *in vitro* redundant enzymes PurN and PurT are both essential for systemic  
 3 infection of mice in *Salmonella enterica* serovar Typhimurium

4  
 5  
 6 Lotte Jelsbak<sup>1,2,†</sup>, Mie I.B. Mortensen<sup>1†</sup>, Mogens Kilstrup<sup>3</sup>, John E. Olsen<sup>1\*</sup>

7  
 8 1. Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of  
 9 Copenhagen, Denmark.  
 10 2. Department of Science, Systems and Models, Roskilde University, Denmark,  
 11 3. Department of Systems Biology, Danish Technical University, Denmark.

12  
 13 \*: Corresponding author  
 14 E-mail: [jeo@sund.ku.dk](mailto:jeo@sund.ku.dk)

15  
 16 <sup>†</sup>These two authors contributed equally to the work.

17  
 18 Abstract number of words: 246  
 19 Number of figures: 1  
 20 Number of Tables: 4  
 21 Number of words in main text (references not included): 5992  
 22  
 23 Running title:  
 24 PurN/PurT and *S. Typhimurium* infection

25 **Abstract**

26 Metabolic enzymes show a high degree of redundancy, and for that reason they are generally  
27 ignored when searching for novel targets for anti-infective substances. The enzymes PurN and  
28 PurT are redundant *in vitro* in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), where  
29 they perform the third step in the purine synthesis. Surprisingly the results of the current study  
30 demonstrated that single gene deletions of each of the genes encoding these enzymes caused  
31 attenuation (competitive infection index < 0.03) in mouse infections. While the  $\Delta purT$  mutant  
32 multiplied as fast as the wild type strain in cultured J774A.1 macrophages, net multiplication of the  
33  $\Delta purN$  mutant was reduced by approximately 50 % in 20 hours. The attenuation of the  $\Delta purT$   
34 mutant was abolished by simultaneous removal of the enzyme PurU, responsible for formation of  
35 formate, indicating that the attenuation was related to formate accumulation or wasteful  
36 consumption of formyl-tetrahydrofolate by PurU. In the process of further characterization, we  
37 disclosed that *in vivo* the enzyme-complex GCV was the most important for formation of C-1 units  
38 *in vivo* (CI:  $0.03 \pm 0.03$ ). In contrast, GlyA was the only important enzyme for the formation of C-  
39 1 units *in vitro*. The results with the  $\Delta gcvT$  mutant further revealed that formation of serine by  
40 SerA and further conversion of serine into C-1 units and glycine by GlyA was not sufficient to  
41 ensure C-1 formation in *S. Typhimurium in vivo*. The study calls for re-investigations of the  
42 concept of metabolic redundancy in *S. Typhimurium in vivo*.

43

## 44 Introduction

45 *Salmonella enterica* is a common cause of food borne disease worldwide. Annually, more than 93  
46 million people have been estimated to suffer from non-Typhoid salmonellosis, and more than  
47 155.000 people succumb to the disease (1). Infection with the serovar *S. Typhimurium* in mice  
48 does not cause diarrhoea, but results in a systemic, life-threatening condition, where bacteria  
49 predominantly localize in cells of the immune system in the liver and spleen. For this reason, *S.*  
50 *Typhimurium* infection of mice is used as a model for systemic salmonellosis, including infection  
51 with the host-specific serovar *S. Typhi* (2).

52  
53 The diversity of intra-cellular and extra-cellular host-niches occupied by *S. Typhimurium* is  
54 reflected in a high-degree of metabolic flexibility (3). This flexibility is achieved through component  
55 and systems-level redundancy in the metabolic network (4), and recent years have seen several  
56 studies of *S. Typhimurium* in order to understand the importance of metabolic redundancy for its  
57 pathogenic life style (5-8). These studies have used genome scale metabolic modelling to predict  
58 essential and combined-lethal metabolic reactions; the latter group consists of two or more non-  
59 essential reactions, which, when considered as one unit, are found to be essential. Such  
60 combinations are referred to as minimal cut-sets in metabolic modelling (9).

61  
62 A list of 102 cut-sets of metabolic reactions in *S. Typhimurium* was recently produced using a  
63 novel genome-scale metabolic model. Each cut-set was predicted to be essential for growth in a  
64 modified M9-minimal medium, and the underlying assumption was that blocking such  
65 combinations of reactions would attenuate *S. Typhimurium* during infection. One cut-set was the  
66 combination of reactions carried out by the enzymes PurN (glycineamide-ribonuclease-  
67 transformylase-N) and PurT (glycineamide-ribonuclease-transformylase-T) in the purine

68 biosynthesis pathway (8). A study using a similar approach by Thiele et al. (5) also predicted that  
69 combined blocking of PurN and PurT would be detrimental to growth.

70

71 Purine *de novo* synthesis consists of ten steps, converting phosphoribosyl-pyrophosphate (PRPP)  
72 into inosine-monophosphate (IMP) (10). In protobacteria the third step, which converts 5'-  
73 phosphoribosyl-glycineamide (GAR) into formyl-phosphoribosyl-glycinamide (fGAR), is carried out  
74 by the two enzymes in the predicted cut-set, PurN and PurT, using different formyl donors.  
75 Blockage of this step results in accumulation of GAR and depletion of all down-stream products,  
76 why it is considered essential for the purine synthesis (11). The reactions carried out by PurN  
77 (GART-RXN) and PurT (GARTRASFORMYL2-RXN) are thus textbook examples of functional  
78 redundancy in metabolic reactions, and the prediction as a cut-set in *S. Typhimurium* was not  
79 surprising.

80

81 PurN and PurT are not isoenzymes because PurN, which is found in both prokaryotes and  
82 eukaryotes (10,12), obtains the formyl-group for generation of fGAR from formyl-tetrahydrofolate  
83 (fTHF), while the prokaryote-specific enzyme PurT uses formate (13) (Fig 1A). As seen in Fig 1B,  
84 PurN and PurT create a link between purine synthesis and the folate Carbon-1 (C-1) metabolism,  
85 where essential C-1 units are formed (14). Aside from delivering carbon-2 and carbon-8 in purine  
86 synthesis, the folate metabolism delivers methyl-groups for the amino acid methionine and the  
87 deoxynucleotide dTMP. PurT is unique in that it uses formate as C-1 donor. Under anaerobic  
88 conditions formate is formed from pyruvate in the oxygen sensitive pyruvate formate lyase (PFL)  
89 reaction (15), but under aerobic conditions, formate is solely derived from fTHF in a reaction  
90 catalysed by the PurU enzyme (16) (Fig 1A). If the PurT enzyme does not use formate, it is a  
91 dead-end product in aerobic metabolism, and it cannot be fuelled back into the C-1 metabolism  
92 (17). Like in *E. coli* (14,18), C-1 units for amino acid and purine synthesis are produced by the

93 GlyA reaction during conversion of serine into glycine, and from degradation of glycine to ammonia  
94 and carbon dioxide by the glycine cleavage system (GCV) (Fig 1B).

95

96 Mutants that lack *purN* or *purT*, or both genes, have been constructed and characterized in *E. coli*  
97 (11). *purN* and *purT* mutants grew slightly slower than their parental strains in minimal medium  
98 under both aerobic and anaerobic conditions. Interestingly, growth of the *purN* mutants was  
99 inhibited by addition of glycine under aerobic, but not under anaerobic conditions, suggesting that  
100 the effect of glycine was due to limiting formate production. A kinetic analysis of the purified PurU  
101 enzyme offered an explanation for this phenomenon, as its hydrolase activity was severely  
102 inhibited by glycine (18). In the *purN* mutant, glycine inhibition of formate production by PurU thus  
103 prevented fGAR synthesis by PurT. When the authors found the PurU activity to be activated also  
104 by histidine, they proposed that the PurU enzyme functions as a regulator that balances the folate  
105 intermediates tetrahydrofolate (THF), methylene tetrahydrofolate (mTHF), and formyl  
106 tetrahydrofolate (fTHF) as a function of the glycine and methionine concentrations.

107

108 Currently we lack good knowledge on the *in vivo* metabolism (in the host) of pathogenic bacteria,  
109 even though this arguably is just as important as virulence factors for the ability of the pathogenic  
110 bacterium to carry out the infection (19). This included lack of knowledge on which nutrients the  
111 bacteria can scavenge from the host, which metabolites different bacteria needs to synthesize and  
112 how this differs between different bacteria, different hosts and even different places the bacterium  
113 occupies in the same host during infection. In the absence of experimental data, we rely on  
114 deduction from their *in vitro* (laboratory) growth phenotypes and model simulations. In some  
115 situations we observe good correlations between *in vitro* and *in vivo* phenotypes with respect to  
116 prediction of the ability to carry out the infection (8), but sometimes this is not the case, probably  
117 because functional auxotrophy may arise when metabolites are present in levels below an

118 important threshold during infection of the host, resulting in unforeseen regulatory effects. Under  
119 such conditions, predicted redundant reactions may turn out to be non-redundant. In the current  
120 study, we show that the universally acknowledged redundant enzyme-pair, PurN/PurT does not  
121 show functional redundancy in *S. Typhimurium* during infection of mice. Rather, each enzyme is by  
122 itself essential for infection. Likewise the redundant enzyme pair MetE/MetH was shown to be non-  
123 redundant during mouse infection. An important message from this study is that well-established  
124 pairs of redundant enzymes may be functionally non-redundant *in vivo* and cannot *a priori* be  
125 classified as redundant based upon metabolic modelling. So there is a dire need to reinvestigate  
126 the concept of metabolic redundancy in *Salmonella* and other pathogenic bacteria in the *in vivo*  
127 situation.

128

## 129 **Material and Methods**

130

### 131 **Bacterial strains**

132 *S. Typhimurium* 4/74 was used as wild-type strain and for the construction of mutant strains by  
133 lambda red mediated re-combination (20), essentially as previously described (8) (Table 1).  
134 Mutated alleles were transformed to a clean wild type *S. Typhimurium* 4/74 background by  
135 P22HT105/int<sup>20</sup> mediated transduction, as described (8). Transduction was also used to construct  
136 double and triple mutants. For construction of triple mutants, the resistance marker, normally  
137 kanamycin, was flipped out using the FLT system as described (20), while each gene contained a  
138 different antibiotic cassette in the double mutants. Primers for mutant construction are listed in  
139 Supplementary Material, Table S1, together with primers used to control the mutations, generally  
140 by two PCR-reactions, one targeting the inserted antibiotic resistance gene and the flanking  
141 regions of the desired genes and one targeting both flanking regions.

142

143 Genetic complementation was obtained by PCR-amplification of the relevant gene and subsequent  
144 cloning into the low copy number plasmid pACYC177, followed by transformation of the resulting  
145 plasmid into the mutant strain, essentially as described (21). Primers are listed in Supplementary  
146 Material, Table S1. Restriction enzymes XhoI and BamHI were used for cloning according to the  
147 manufacturer's recommendation (Thermo Scientific, by, Denmark), and constructs were verified  
148 by restriction analysis and sequence analysis.

149

#### 150 Culture and growth conditions

151 Growth of bacteria in rich media was done in Difco™ Lysogeny broth, Lennox (Becton, Dickinson  
152 and Company, Albertslund, Denmark) and on LB agar plates (Becton, Dickinson and Company,  
153 Albertslund, Denmark), and growth in minimal medium was carried out in M9-broth (2 mM MgSO<sub>4</sub>,  
154 0.1 mM CaCl<sub>2</sub>, 0.4 % glucose, 8.5 mM NaCl, 42 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 18.6 mM NH<sub>4</sub>Cl).  
155 Chloramphenicol 10 ug/ml, kanamycin 50 ug/ml, glycine 0.55 mg/ml, serine 0.55 mg/ml and  
156 methionine 0.55 mg/ml (Sigma, Denmark), were added when appropriate.

157

158 Growth phenotypes of mutants were determined by first growing the bacterium overnight in LB  
159 flasks at 37 °C with shaking (200 rpm). This culture was diluted 40 fold into 0.2 ml M9 and growth  
160 was followed for 24 hours at 37 °C with shaking (250 rpm) by OD<sub>600</sub> measurement each 15  
161 minutes in a BioScreen CTM format with biological triplicates and technical replicates. Wild type  
162 strain and blank wells were included as controls. Growth curves were extracted using Excel  
163 (Microsoft, SanDiego, USA) and OD<sub>600</sub> values were corrected for the blank controls.

164

#### 165 Microscopic appearance of bacteria

166 The microscopic appearance of bacteria was determined by phase contrast microscopy at fixed  
167 time points and conditions using an AxioCamHR4 phase-contrast microscope. Three hundred



168 individual cells were observed to determine the most common cell morphology. Continuous  
169 observations of cell morphology during growth in LB media at 37 °C was done using oCelloScope  
170 bright field camera (magnification of approximately 200x and resolution of 1.3 µm) as reported  
171 (22).

172

### 173 Macrophage survival experiments

174 Survival and multiplication inside J774 macrophages were measured as described (21). *S.*  
175 Typhimurium 4/74 was used as reference wild type, and  $\Delta$ *ssaI* mutant in the 4/74 background  
176 (21) was used as negative control. Deletion of this gene renders *S. Typhimurium* incapable of intra  
177 cellular replication (23). Briefly, MOI of infection was 5 and 25 minutes was allowed for the initial  
178 uptake of bacteria, whereafter gentamicin (100 ug/ml) was added for one hour and then replaced  
179 with 25 ug/ml for the rest of the experiments. CFU counts with three or four biological repeats and  
180 with technical duplicates were obtained at the point of the first addition of gentamicin, and after 1  
181 hour, 2 hours and 21 hours of incubation with this drug.

182

### 183 Mouse infections

184 Measurement of infection efficacy was performed using a systemic model of infection in C57/BL6  
185 female mice (Taconic, Denmark). The competitive challenge model (24) where wild type and  
186 mutant strain were given together in the same mouse was used, as described (8). Briefly, mice  
187 were inoculated by the I.P. route with 0.1 ml of an approximately 1:1 mixture of wild type and  
188 mutant strains in PBS. The inoculum was standardized to contain a challenge dose of  $5 \times 10^3$   
189 bacteria of each strain using CFU measurements. The exact amount of each strain in the inoculum  
190 was determined by plating serial dilutions on LB plates. The ratio between wild type and mutant  
191 bacteria in the spleen was determined 6 days post-inoculation by plating dilution series on LB agar  
192 and subsequently determining the resistance (chloramphenicol or kanamycin resistance) of 100

193 colonies. Sensitive bacteria corresponded to wild type and resistant bacteria to mutants. The  
194 competitive index was calculated as the mutant/wild type ratio of the spleen count versus the  
195 mutant/wild type ratio of the inoculum. Severely affected mice were humanely killed. If the spleen  
196 of such mice contained  $>10^5$  CFU *Salmonella* this was expected to be the cause of the disease, and  
197 colony counts of such mice were included in the competitive index scoring.

198

### 199 **Ethical statement**

200 Mice challenge experiments were conducted with permission to the senior author from the Danish  
201 Animal Experiments Inspectorate according to Danish by-law No. 474 of 15<sup>th</sup> May 2014 (license  
202 number: 2009/561–1675).

203

### 204 **Statistical analysis**

205 Statistical differences between wild type and mutant strains in CFU, and in virulence measured in  
206 mice, was determined using GraphPad Prism®, version 5.0 (GraphPad software, Town, USA) with  
207 one-sample t-test analysis. Grubb's outlier test was performed to exclude outliers with a  
208 significance of 0.05.

209

## 210 **Results**

211

### 212 **PurN and PurT are redundant *in vitro* but not *in vivo*.**

213 PurN and PurT have been reported to be redundant for growth of *E. coli*, although *purN* and *purT*  
214 mutants grow somewhat slower than the wild type (11). Single mutants in *S. Typhimurium* 4/74  
215 created by deletion of the *purN* and *purT* genes grew as well as the parental wild type strain in  
216 minimal medium (WT:  $\mu = 0.37 \pm 0.017$ ,  $\Delta purN$ :  $\mu = 0.35 \pm 0.01$  and  $\Delta purT$ :  $\mu = 0.36 \pm 0.02$ ), while

217 the double mutant did not grow. The growth determination of the  $\Delta purN$  mutant is shown in  
218 Supplementary Figure S1 to demonstrate the way specific growth rates were determined. When  
219 grown in rich media (LB), the double mutant had no growth defect (data not shown). This  
220 corresponds to PurN and PurT being redundant for growth in minimal medium *in vitro*.

221

222 When the mutants were analysed for their virulence in mice, using competitive challenging  
223 experiments, both single and double mutants were severely attenuated, showing competitive  
224 indexes (CIs) below 0.03. Virulence of single mutants could be raised to normal levels by  
225 complementation with wild type genes *in trans* (Table 2), showing that the attenuation was due to  
226 lack of PurN and PurT, respectively. Thus, in the infection situation, one or more factors are  
227 limiting for growth in an enzyme-specific manner for the presumed redundant enzyme-pair  
228 PurN/PurT, clearly showing that they are not redundant *in vivo* during infection of mice.

229

230 **Mutation of *purN* but not *purT* attenuates the strain during interaction with**  
231 **cultured macrophages.**

232 Interaction with macrophages is an important step in the development of systemic salmonellosis in  
233 mice, and the majority of mutants that fail to grow in cultured macrophages have turned out to be  
234 attenuated during mice infection (25). To investigate the ability to grow in macrophages, we  
235 challenged cultured J774A.1 macrophages with  $\Delta purN$  and  $\Delta purT$  mutants. As seen in Table 2, the  
236 WT strain was found to multiply  $9.7 \pm 2.5$  times in 20 hours. This equals approximately seven-hour  
237 generation time, and corresponds to estimates for multiplication of *S. Typhimurium* in the spleen  
238 of mice *in vivo* (26). In contrast, the  $\Delta purN$  mutant only multiplied  $4.4 \pm 3.5$  times, which was  
239 significantly less than the wild type strain ( $p=0.03$ ). The  $\Delta purT$  mutant resembled the wild type  
240 strain and multiplied  $8.0 \pm 5.6$  times, suggesting that the attenuation in virulence caused by the  
241 deletion of *purT* was not related to interaction at the macrophage level, while attenuation of the

242  $\Delta purV$  mutant might be related to replication in cells of this type. The double mutant was severely  
243 attenuated, and the resulting number of bacteria after 20 hours was reduced 70% compared to  
244 the number of cells taken up by the macrophages (Table 2). The control strain, *S. Typhimurium*  
245 4/74 lacking *ssaV*, encoding an effector protein of the type three secretion system, SPI-2,  
246 associated with intracellular multiplication (23), multiplied  $2.1 \pm 0.6$  times, which was within the  
247 expected range, showing that the assay was performing as expected. The differences in  
248 multiplication were not caused by different starting concentrations, since all strains were taken up  
249 by the macrophages to the same degree (data not shown).

250

## 251 Cooperation between PurT and PurU *in vitro*, in macrophages and during mouse 252 infection

253

254 Conversion of GAR to fGAR by the PurT enzyme requires free formate, provided by PurU (16). If  
255 the attenuation caused by deletion of *purT* was related to accumulation of formate, then deletion  
256 of *purU* in a  $\Delta purT$  background should eliminate the attenuation.

257

258 Contrary to  $\Delta purT$ , a  $\Delta purU$  mutant showed no growth defect in *E. coli* (16). Interestingly, the  
259 opposite was observed in *S. Typhimurium*. The  $\Delta purT$  mutant was not growth arrested, whereas a  
260 *purU* deletion in *S. Typhimurium* strain 4/74 resulted in a reduced growth in M9 minimal medium  
261 ( $\mu = 0.25 \pm 0.002$ ; p-value compared to WT strain  $< 0.05$ ). Complementation by providing the *purU*  
262 gene *in trans* did not restore wild type growth; on the contrary, it reduced growth rate further ( $\mu =$   
263  $0.15 \pm 0.002$ ) (Table 2). A possible explanation is that provision of PurU *in trans* on a plasmid  
264 results in too high enzyme levels and excessive conversion of fTHF into THF and formate. A *purT*  
265 deletion in the  $\Delta purU$  mutant restored normal growth ( $\mu = 0.38 \pm 0.01$ ). This showed that the slow  
266 growth rate of a  $\Delta purU$  mutant was not related to lack of substrate for PurT, and that the growth

267 attenuation of the  $\Delta purT$  mutant probably was caused by formate accumulation or wasteful  
268 conversion of fTHF to THF, which disappears when PurT and PurU are absent in the same  
269 bacterium.

270

271 In macrophage experiments the  $\Delta purU$  mutant was significantly attenuated in multiplication over  
272 20 hours ( $4.6 \pm 2.5$ ;  $p < 0.05$ ). The  $\Delta purU$  mutant was also significantly attenuated in mouse  
273 virulence, albeit not to a level that resembled  $\Delta purN$  and  $\Delta purT$  mutants (Table 2). In accordance  
274 with the phenotype seen during growth in M9 medium, a completely restored virulence of the  
275  $\Delta purT \Delta purU$  double mutant was seen in the mice assay (CI: 1.6) (Table 2).

276

#### 277 Glycine but not serine is available for C-1 production during mouse infection

278 Purine and C-1 metabolism are closely linked, and the reason that PurN and PurT are not  
279 functionally redundant *in vivo* during infection of mice could be related to the need to secure  
280 sufficient purine synthesis and C-1 metabolism to occur concurrently. Detailed investigations of the  
281 role of C-1 metabolism of *S. Typhimurium* in virulence have not been reported, and we therefore  
282 undertook a series of characterizations of mutants in the *S. Typhimurium* serine and glycine  
283 metabolism.

284

285 We have previously predicted and validated by challenge experiments that a  $\Delta serA$  mutant, which  
286 is unable to synthesize serine and therefore rely on uptake of serine or production of serine from  
287 glycine for serine production (see Fig 1B), is fully virulent in mice (8). Others have likewise  
288 predicted by *in silico* modelling that SerA is not needed for growth *in vivo* (5). From this we  
289 previously concluded that serine was most likely taken up from the host environment, but that  
290 further studies were needed to understand the relative contribution of GlyA and the glycine  
291 cleavage enzyme, GVC, in the phenotype of the  $\Delta serA$  mutant (8). In the present study we

292 analysed a  $\Delta gcvT$  mutation that cannot convert glycine into C-1 units and CO<sub>2</sub> (see Fig 1B).  
293 Whether the  $\Delta gcvT$  mutation was present alone or together with the  $\Delta serA$  mutation, virulence  
294 was reduced significantly. The strain multiplied like the wild type strain in cultured macrophages,  
295 suggesting that the limiting step in infection was not intracellular multiplication in this cell type  
296 (Table 3). The results show that exogenous glycine, and not serine as previously believed, was  
297 available during infection. If serine had been available in sufficient amounts for synthesis of glycine  
298 and C-1 units, then the  $\Delta gcvT$  mutation would have had no effect. This appears to be the situation  
299 *in vitro*, since the  $\Delta gcvT$  mutant grew as well as the wild type strain in M9 medium ( $\mu =$   
300  $0.31 \pm 0.21$ ), while the  $\Delta glyA$  mutant was severely growth attenuated. This phenotype could be  
301 reversed by addition of the wild type *glyA* gene *in trans*, and addition of glycine to the minimal  
302 medium also restored growth partially (Table 3). The wild type 4/74 strain was not affected by  
303 addition of glycine to the medium (data not shown). These observations suggest that *in vitro* GlyA  
304 is the enzyme that is most important for production of C-1 units, while the glycine cleave system  
305 has this role *in vivo*, and it underscores the difficulty in predicting *in vivo* importance from *in vitro*  
306 growth phenotypes. We can also conclude from the  $\Delta gcvT$  single mutant, that serine production  
307 through the SerA enzyme (present in the  $\Delta gcvT$  single mutant) and subsequent conversion to  
308 glycine and C-1 units by the GlyA enzyme (also present) is not sufficient to support virulence of  
309 *Salmonella* Typhimurium 4/74 in the mouse model.  
310  
311 These conclusions were corroborated by the analysis of the  $\Delta glyA$  mutant, which was only partly  
312 reduced in virulence (Table 3). If glycine were normally synthesized from serine through the GlyA  
313 enzyme, then the mutation would have had severe consequences. As expected, the introduction of  
314 a  $\Delta serA$  mutation (in itself dispensable) in the  $\Delta glyA$  strain (partly avirulent) resulted in total  
315 avirulence, because now the cell had no means of obtaining serine for protein synthesis. For the  
316 same reason, Thiele et al. (5) predicted this combination to be lethal in *S. Typhimurium*. Deletion

317 of the *gcvT* gene in the  $\Delta$ *glyA* background increased attenuation even further than the two single  
318 mutations, and no colonies were obtained from mice challenged with this strain. If for statistical  
319 purposes one assumed one colony per mice, this would not have been significantly different from  
320 the  $\Delta$ *gcvT* mutant on its own, but significantly more attenuated than the  $\Delta$ *glyA* mutant (Table 3).  
321 The observation was expected, because now the production of C-1 units for purine and methionine  
322 synthesis was totally blocked. In macrophages, growth of the  $\Delta$ *glyA* mutant was highly impaired,  
323 and a wild-type *glyA* gene provided *in trans* complemented this phenotype (Table 3), suggesting  
324 that either glycine biosynthesis or formation of serine from glycine was a limiting factor for *S.*  
325 Typhimurium growth in macrophages, but also that the lower multiplication rate does not lead to  
326 total avirulence in mice (CI: 0.3).

327  
328 The results with the *glyA* mutant to some extent contradicted a previous report by us (8), where  
329 we concluded that the  $\Delta$ *glyA* deletion mutant was more attenuated in mice than reported here.  
330 Upon microscopic analysis of the different mutants we discovered a possible reason for this  
331 discrepancy. The  $\Delta$ *glyA* deletion, and deletion of *glyA* in combination with *gcvT*, resulted in a  
332 mixture of normally shaped and elongated bacteria (Supplementary material Fig S2. The altered  
333 relation between the optical density and the concentration of colony forming units of the  $\Delta$ *glyA*  
334 mutant cultures may have led us to the overestimation of the importance in virulence in the  
335 previous study where we used OD-values to prepare the inoculum, because the elongated cells  
336 resulted in a lower mutant-to-wild type ratio in the input pool. In the present study, the infections  
337 were performed with a one-to-one ratio based upon number of colony forming units, eliminating  
338 this problem. Elongation of cells could be eliminated by complementation *in trans* (data not  
339 shown), showing that the cell division was somehow affected by elimination of the GlyA reaction.  
340 We did not enquire further into this interesting phenotype in the present study.

341

342 We showed above that virulence attenuation of the  $\Delta purT$  mutant was probably associated to  
343 imbalance in formate or fTHF conversion. To investigate whether lack of THF production affected  
344 the phenotype of mutants in C-1-metabolism, we characterized a number of combined purine  
345 synthesis and C-1 metabolism mutants. All four combinations of  $\Delta serA$  or  $\Delta glyA$  mutations with  
346 mutation in  $purN$  and  $purT$  were growth attenuated to the same extent as the single  $\Delta serA$  or  
347  $\Delta glyA$  mutants *in vitro* (combinations with  $\Delta purN$ :  $\mu = 0.00$  ( $\Delta serA$ ) and  $0.04 \pm 0.003$  ( $\Delta glyA$ );  
348 combinations with  $\Delta purT$ :  $\mu = 0.00$  ( $\Delta serA$ ) and  $0.03 \pm 0.00$  ( $\Delta glyA$ )). From this we concluded that  
349 there was no significant effect of the extra deletion of  $purN$  and  $purT$  in the  $\Delta serA$  and  $\Delta glyA$   
350 mutants *in vitro* even though the competing use of methylene-THF for purine biosynthesis was  
351 prevented. We also tested the  $\Delta purN/\Delta glyA$  strain in the mouse model and this strain was not  
352 significantly different from the  $\Delta purN$  mutant on its own (CI:  $0.02 \pm 0.02$ ), while the  $\Delta purT/\Delta serA$   
353 mutant apparently was less attenuated than the  $\Delta purT$  strain (CI:  $0.17 \pm 0.13$ ), however the  
354 difference was not statistically different from the  $\Delta purT$  strain on its own ( $p > 0.05$ ). Together these  
355 experiments indicated little or no overlap between the two systems.

356

### 357 **Synthesis of methionine from homocysteine and C1-THF *in vivo*.**

358 Methionine synthesis is also interlinked with the purine synthesis through the common pool of  
359 mTHF and THF (Fig 1B), and we therefore also considered its importance for the results obtained  
360 with *S. Typhimurium* lacking  $PurN$  or  $PurT$ . Methionine is synthesized by two homocysteine  
361 methylating enzymes, MetE and MetH (27). The apparent redundancy of the reaction should  
362 enable the strain to synthesize methionine even when either the *metE* or the *metH* gene was  
363 mutated, and previous predictions of redundancy in *S. Typhimurium* has predicted that these two  
364 enzymes form a cut-set (6). However, while the MetE enzyme is functional under aerobic growth,  
365 the MetH enzyme requires vitamin B12, which is only synthesized in *S. Typhimurium* under  
366 anaerobic conditions (28). The  $\Delta metE$  mutant was found to be highly growth retarded ( $\mu =$



0.09±0.03), most probably because the bacterium then relies on the anaerobic enzyme, MetH. Interestingly, however, also a  $\Delta metH$  mutant was found to grow with a lower growth rate than the wild type in M9 medium ( $\mu = 0.20 \pm 0.01$ ), suggesting that the MetH enzyme is important under the growth condition tested (Table 4). As expected, a  $\Delta metE \Delta metH$  double mutant did not grow in M9 minimal medium, since no methionine is available to this strain for protein synthesis. This phenotype could be complemented by addition of methionine to the medium ( $\mu = 0.31 \pm 0.02$ ), a step that did not affect growth of the wild type strain (data not shown) and showing that methionine biosynthesis is dispensable, when methionine can be taken up from the environment. The same was the case for the growth defects of the  $\Delta metH$  single mutant ( $\mu = 0.35 \pm 0.03$  with methionine supplied). When the *metE* gene was supplied in trans to the  $\Delta metE \Delta metH$  double mutant, the growth was fully restored ( $\mu = 0.24 \pm 0.03$ ) to the level of the  $\Delta metH$  single mutant, showing the importance of the MetH enzyme during aerobic growth even with the MetE protein expressed from a multicopy plasmid. Addition of methionine to the  $\Delta metE$  mutant in M9 medium likewise raised the growth rate ( $\mu = 0.20 \pm 0.01$ ). A triple mutant, where the double *metE metH* mutations were combined with mutation of *purT* did not grow in M9 media, corresponding to the phenotype of the  $\Delta metE \Delta metH$  mutant on its own. Addition of methionine restored growth completely in this mutant ( $\mu = 0.35 \pm 0.01$ ), which also corresponded to the  $\Delta metE \Delta metH$  mutant on its own. Taken together the results were interpreted as no or very little interaction between PurT and MetH/MetE, and vice versa.

The  $\Delta metH$  mutant was slightly but significantly attenuated during mouse infection with a competitive index of 0.4 ( $p < 0.001$ ), while the  $\Delta metE$  single mutant and the  $\Delta metE \Delta metH$  double mutants were totally avirulent (Table 3). Although the *metE* gene *in trans* fully complemented the growth phenotype of the double mutant to the level of the  $\Delta metE$  mutant in M9 medium, the presence of the *metE* gene on a multicopy gene did not render the double mutant virulent at all.

392 Methionine appears to be available in macrophages to some extent, as the  $\Delta metE/\Delta metH$  double  
393 mutant could multiply three-fold within twenty hours in cultured macrophages (Table 4). While  
394 methionine is available in macrophages, we may conclude from the infection studies, that it is not  
395 available during other phases of mouse infection. Elimination of *purT* in the double mutant  
396 background reduced net multiplication rate in macrophages significantly ( $0.73 \pm 0.29$ ), suggesting a  
397 synergistic effect of the mutations at this level.

398

## 399 Discussion

400 The main aim of this study was to determine the contribution of the *in vivo* redundant enzymes  
401 PurN and PurT to virulence in *S. Typhimurium*, and when realizing that they were not redundant *in*  
402 *vivo* during infection of mice, to elucidate possible reasons for this. In the broader perspective, the  
403 study illustrates that one cannot safely assume that *in vitro* redundancy between enzymes is  
404 followed by a similar *in vivo* redundancy during infection of mice. Another important observation,  
405 based on the *in vitro* growth experiments, is that even though *E. coli* and *S. Typhimurium* share  
406 the basic architecture of the metabolic systems we have investigated, the growth phenotypes  
407 associated with knock out of a gene cannot always be assumed to be the same. This notion has  
408 recently been highlighted in another publication, dealing with thiamine biosynthesis in *E. coli* (29).

409

410 The concept of redundancy has mainly been understood from studies of *E. coli* K12. When it grows  
411 on glucose as the sole carbon source, more than 80 of the 227 metabolic enzymes are non-  
412 essential (30). Redundancy may be a trade-off between efficiency and robustness, and organisms  
413 with a broad niche repertoire show the highest degree of redundancy. Therefore, redundancy has  
414 been interpreted as a mechanism that supports niche adaptation (31). Others, however, consider  
415 redundancy as a way to withstand detrimental mutations (32). Our observations with PurN and  
416 PurT, and MetE and MetH for that matter, suggest that some pairs of redundant enzymes may be

417 artefacts of studying bacteria in test tubes, and that the two enzymes are maintained because  
418 they are both essential, possibly at different steps in the normal live cycle (infection steps in the  
419 case of pathogenic bacteria). This corresponds mostly to the niche adaptation theory, since the  
420 likely explanation for both enzymes being essential is that *Salmonella* goes through a series of  
421 different environments (niches) in the infection process. However, in the niche adaptation theory,  
422 the need for both of the enzymes is not absolute.

423

424 The total avirulence of both PurN and PurT mutants could be complemented by addition of the  
425 respective enzyme encoded from a plasmid *in trans*. This proved that the attenuation was related  
426 to the lack of the specific enzymes. It is well known that purine biosynthesis is required for  
427 intracellular multiplication, and purine biosynthesis mutants have been employed as live vaccines  
428 against *S. Typhi* (33) and *S. Typhimurium* (34), as well as *Mycobacterium tuberculosis* (35),  
429 *Brucella melitensis* (36) and *Francisella tularensis* (37). In light of this it was not surprising that  
430 the double  $\Delta purN/\Delta purT$  mutant was attenuated and unable to multiply inside macrophages.  
431 However, previous studies on putative targets genes for attenuated live vaccines have ignored the  
432 3<sup>rd</sup> step in the biosynthesis of purines, catalysed by PurN and PurT, due to the recognized  
433 redundancy between the enzymes *in vitro*. Based on our results, deletion mutants in either PurN or  
434 PurT are likely also to be good vaccine candidates against *Salmonella*.

435

436 Studies of multiplication ability in cultured macrophages is a sensitive method to identify virulence  
437 attenuation in *Salmonella*, and Leung and Finlay (38) showed that mutants that could not multiply  
438 in cultured macrophages were also avirulent in mice. The opposite, however, is not necessarily the  
439 case. The avirulent PurT mutant was not critically affected in propagation in the intracellular  
440 environment of J774A.1 macrophages. This suggested that the interaction with this cell type was  
441 not the limiting point for the PurT mutant. Contrary to the  $\Delta purT$  mutant, the  $\Delta purN$  mutant grew

442 poorly or was killed more quickly than wild type inside macrophages (these two things cannot be  
443 separated by the assay we used), suggesting that macrophage survival/growth could be one of the  
444 critical points in progression in the host for this mutant. Recent studies have shown that one  
445 should be careful not to over-interpret results from experiment with cultured macrophages, and  
446 especially not to draw conclusions on mechanism of virulence from such studies (39). Also it  
447 should be noted that the J774 cells have a different genetic background (BalB/c) than the  
448 mouse strain used, and even though they are both *slr*<sup>-/-</sup> (N-ramp<sup>neg</sup>), this may make it difficult  
449 to compare the *in vivo* and the *in vitro* situation directly, Our results with macrophages should  
450 thus be interpreted with caution.

451

452 Studies using a mutant in the essential *S. Typhimurium purG* gene (also termed *purL*) have shown  
453 that this purine auxotroph strain fail to repair DNA damage caused by Reactive Oxygen Species in  
454 the phagosome environment of the macrophage, and that this can explain the inability of the  
455 mutant to multiply inside macrophages (40). Our results suggest that PurN has sufficient activity  
456 within macrophages to ensure that DNA damage can be repaired in the absence of PurT, while  
457 PurT cannot ensure wild type propagation in macrophages in the absence of PurN. The reduction  
458 in net growth of the PurN mutant with approximately 50 % corresponds to previous observations  
459 on net growth rate of a  $\Delta purH$  mutant in mouse spleens (26). The results indicated that intra  
460 cellular propagation was the rate-limiting step causing attenuation of the *purN* mutant, and also  
461 that purines cannot be supplied in sufficient amounts from external sources during macrophage  
462 infection. A recent study has shown that the  $\Delta purH$  mutant grows with two distinct populations  
463 with respect to location and growth rate in the spleen of mice (26), probably reflecting that the  
464 growth happens in two compartments with two different demands for purine synthesis, and it  
465 would be interesting to investigate whether the  $\Delta purN$  mutant also grows as two separate  
466 populations inside macrophages.

467

468 Purine synthesis has been found to be marginally (down) regulated when *S. Typhimurium* grows  
469 inside cultured macrophages, and PurN and PurT are regulated to the same degree, although only  
470 PurN was significant different from the control by the statistical analysis used in the study (41).  
471 The reference condition was growth of opsonized bacteria in cell culture media, and the  
472 observation is therefore difficult to compare to the present study, where bacteria were grown in LB  
473 media prior to macrophage challenge; however, it indicates that *S. Typhimurium* requires as much  
474 or slightly more purine *de novo* biosynthesis to grow in cell culture medium as it requires for  
475 growing inside macrophages.

476

477 With current techniques it is not possible to determine the exact amounts of important  
478 intermediates in the purine biosynthesis, such as THF species, in bacteria during infection. In order  
479 to understand why both enzymes were essential and whether this was related to imbalance on the  
480 consumption and production of THF species, we chose a mutant-based approach, where relevant  
481 genes were deleted in different combinations. Our studies with  $\Delta purU$  and  $\Delta purT$  strains strongly  
482 suggested that a main reason for attenuation of the *purT* mutant was accumulation of formate or  
483 wasteful use of fTHF by PurU, since the double  $\Delta purT/\Delta purU$  mutant was fully virulent. For this to  
484 make sense, the metabolism should be aerobic, since this is the condition where formate  
485 production by PurU is a dead end product in the absence of PurT (16). In this sense, our  
486 observation supports a recent study, showing that the environment perceived by *Salmonella*, when  
487 it resided in cells of the monocyte line in the spleen, is aerobic, because *Salmonella* exclusively  
488 resides in the red pulp in close proximity to erythrocytes (26). It still needs to be determined  
489 whether formate accumulation or wasteful fTHF, or both, are the important factor, and to detect  
490 the exact point in infection, where this may be the case. The fact that the mutant without PurT

491 could propagate in cultured macrophages suggested that formate accumulation and/or wasteful  
492 fTHF consumption is not a problem inside such cells.  
493  
494 The current study also made important observations with regard to the role of C-1-metabolism in  
495 virulence. Based on *in vitro* experiments, the glycine cleavage enzyme, GCV, has been estimated  
496 to contribute less to formation of C-1 units in *E. coli* than the hydroxyl-methyltransferase-enzyme  
497 encoded by *glyA* (14). The growth experiments in the current study shows that *S. Typhimurium* *in*  
498 *vitro* shows the same balance between the two enzymes, since the  $\Delta gcvT$  mutant was not growth  
499 attenuated, while the  $\Delta glyA$  mutant did not grow. *In vivo*, however, the situation was totally  
500 opposite. The  $\Delta glyA$  strain was only partly attenuated, while the  $\Delta gcvT$  mutant was highly  
501 attenuated and colonies were rarely isolated from any infected animals. Interestingly, concurrent  
502 mutation in *serA* did not change this, which showed that contrary to previously expected (8),  
503 exogenous glycine, and not serine must be available to *Salmonella* during infection. Else, glycine  
504 and C-1 units could have been formed by *glyA* from serine, and then the  $\Delta gcvT$  mutation would  
505 have had no effect (Figure 1B). When  $\Delta purN$  or  $\Delta purT$  mutations were combined with mutation of  
506 *serA* or *glyA*, the *in vitro* phenotypes indicated that the purine synthesis and C-1 metabolism  
507 systems did not interfere significantly with each other. Double mutants caused the same  
508 phenotype as the single gene with the highest influence on the performance of *S. Typhimurium*.  
509 Unexpectedly we discovered that  $\Delta glyA$  mutants form elongated cells when growing *in vitro*.  
510 Filament formation is well known in *Salmonella* as a response to osmotic and cold stress (42,43),  
511 but this phenotype of *glyA* mutation has, to the best of the author's knowledge, not previously  
512 been described in this genus, nor in any of its close relatives. The phenotype could be reversed by  
513 genetic complementation *in trans*, ruling out that it was caused by secondary mutations in genes  
514 of relevance for Z-ring formation.  
515

516 In the process of detailing the link between the purine and methionine metabolism we discovered  
517 that the enzymes MetE/MetH, which are considered redundant (6) also did not show redundancy  
518 during infection. We confirmed that methionine is not taken up from the host during mice  
519 infection, since the  $\Delta metE/\Delta metH$  double mutant was avirulent. It has previously been shown that  
520 mutation of *metC*, which mobilises sulphur for methionine, is essential in *S. Typhimurium* (44), and  
521 *S. Typhimurium* is only one among several pathogens where methionine biosynthesis appears to  
522 be essential during growth in the animal host (45,46). *In vitro*, the double mutant did not grow  
523 either. This phenotype could be complemented by addition of methionine to the medium, showing  
524 that the methionine uptake system can compensate fully for lack of the biosynthesis system during  
525 growth *in vitro*. Provision of *metE* *in trans* restored growth to the level of the  $\Delta metH$  mutant. This  
526 demonstrated that the *metE* mutation can be complemented *in trans* and that MetE and MetH are  
527 not fully redundant; the enzyme MetH, which is associated to anaerobic growth (26), must play a  
528 role during this growth, even though the cultures were shaken. Further studies are needed to fully  
529 understand the reasons for this observation. Like for the C-1 metabolism, we did not find any  
530 indication that purine mutation significantly affected the phenotypes obtained after mutation of  
531 methionine synthesis genes.

532

### 533 Acknowledgement

534 The technical assistance of Tony Bønnelycke and Pia Mortensen is highly appreciated.

535

### 536 References

537

- 538 1. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A,  
539 Hoekstra RM, International Collaboration on Enteric Disease 'Burden of Illness S.

- 540 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin Infect Dis*  
541 **50**:882-889.
- 542 2. **Garcia-del Portillo F.** 2001. *Salmonella* intracellular proliferation: where, when and  
543 how? *Microbes Infect* **3**:1305-1311.
- 544 3. **Becker D, Selbach M, Rollenhagen C, Ballmaier M, Meyer TF, Mann M, Bumann D.**  
545 2006. Robust *Salmonella* metabolism limits possibilities for new antimicrobials. *Nature*  
546 **440**:303-307.
- 547 4. **Wang Z, Zhang J.** 2009. Abundant indispensable redundancies in cellular metabolic  
548 networks. *Genome Biol Evol* **1**:23-33.
- 549 5. **Thiele I, Hyduke DR, Steeb B, Fankam G, Allen DK, Bazzani S, Charusanti P, Chen**  
550 **FC, Fleming RM, Hsiung CA, De Keersmaecker SC, Liao YC, Marchal K, Mo ML,**  
551 **Ozdemir E, Raghunathan A, Reed JL, Shin SI, Sigurbjornsdottir S, Steinmann J,**  
552 **Sudarsan S, Swainston N, Thijs IM, Zengler K, Palsson BO, Adkins JN, Bumann D.**  
553 2011. A community effort towards a knowledge-base and mathematical model of the  
554 human pathogen *Salmonella* Typhimurium LT2. *BMC Syst Biol* **5**:8.
- 555 6. **Steeb B, Claudi B, Burton NA, Tienz P, Schmidt A, Farhan H, Maze A, Bumann D.**  
556 2013. Parallel exploitation of diverse host nutrients enhances *Salmonella* virulence.  
557 *PLoS Pathog* **9**:e1003301.
- 558 7. **Hartman HB, Fell DA, Rossell S, Jensen PR, Woodward MJ, Thorndahl L, Jelsbak L,**  
559 **Olsen JE, Raghunathan A, Daefler S, Poolman MG.** 2014. Identification of potential  
560 drug targets in *Salmonella enterica* sv. Typhimurium using metabolic modelling and  
561 experimental validation. *Microbiology* **160**:1252-1266.
- 562 8. **Jelsbak L, Hartman H, Schroll C, Rosenkrantz JT, Lemire S, Wallrodt I, Thomsen**  
563 **LE, Poolman M, Kilstrup M, Jensen PR, Olsen JE.** 2014. Identification of metabolic



- 564 pathways essential for fitness of Salmonella Typhimurium in vivo. PLoS One  
565 9:e101869.
- 566 9. **Klamt S, Gilles ED.** 2004. Minimal cut sets in biochemical reaction networks.  
567 Bioinformatics **20**:226-234.
- 568 10. **Zhang Y, Morar M, Ealick SE.** 2008. Structural biology of the purine biosynthetic  
569 pathway. Cell Mol Life Sci **65**:3699-3724.
- 570 11. **Nygaard P, Smith JM.** 1993. Evidence for a novel glycinamide ribonucleotide  
571 transformylase in Escherichia coli. J Bacteriol **175**:3591-3597.
- 572 12. **Sampei G, Kanagawa M, Baba S, Shimasaki T, Taka H, Mitsui S, Fujiwara S,**  
573 **Yanagida Y, Kusano M, Suzuki S, Terao K, Kawai H, Fukai Y, Nakagawa N, Ebihara**  
574 **A, Kuramitsu S, Yokoyama S, Kawai G.** 2013. Structures and reaction mechanisms of  
575 the two related enzymes, PurN and PurU. J Biochem **154**:569-579.
- 576 13. **Thoden JB, Firestone S, Nixon A, Benkovic SJ, Holden HM.** 2000. Molecular structure  
577 of Escherichia coli PurT-encoded glycinamide ribonucleotide transformylase.  
578 Biochemistry **39**:8791-8802.
- 579 14. **Plamann MD, Stauffer GV.** 1989. Regulation of the Escherichia coli glyA gene by the  
580 metR gene product and homocysteine. J Bacteriol **171**:4958-4962.
- 581 15. **Knappe J, Sawers G.** 1990. A radical-chemical route to acetyl-CoA: the anaerobically  
582 induced pyruvate formate-lyase system of Escherichia coli. FEMS Microbiol Rev **6**:383-  
583 398.
- 584 16. **Nagy PL, McCorkle GM, Zalkin H.** 1993. purU, a source of formate for purT-dependent  
585 phosphoribosyl-N-formylglycinamide synthesis. J Bacteriol **175**:7066-7073.
- 586 17. **Dev IK, Harvey RJ.** 1982. Sources of one-carbon units in the folate pathway of  
587 Escherichia coli. J Biol Chem **257**:1980-1986.

- 588 18. **Nagy PL, Marolewski A, Benkovic SJ, Zalkin H.** 1995. Formyltetrahydrofolate  
589 hydrolase, a regulatory enzyme that functions to balance pools of tetrahydrofolate and  
590 one-carbon tetrahydrofolate adducts in *Escherichia coli*. *J Bacteriol* **177**:1292-1298.
- 591 19. **Abu Kwaik Y, Bumann D.** 2013. Microbial quest for food in vivo: 'nutritional  
592 virulence' as an emerging paradigm. *Cell Microbiol* **15**:882-890.
- 593 20. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in  
594 *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**:6640-6645.
- 595 21. **Wallrodt I, Jelsbak L, Thomsen LE, Brix L, Lemire S, Gautier L, Nielsen DS,**  
596 **Jovanovic G, Buck M, Olsen JE.** 2014. Removal of the phage-shock protein PspB  
597 causes reduction of virulence in *Salmonella enterica* serovar Typhimurium  
598 independently of NRAMP1. *J Med Microbiol* **63**:788-795.
- 599 22. **Kjeldsen TS, Sommer MO, Olsen JE.** 2015. Extended spectrum beta-lactamase-  
600 producing *Escherichia coli* forms filaments as an initial response to cefotaxime  
601 treatment. *BMC Microbiol* **15**:63.
- 602 23. **Hensel M, Shea JE, Raupach B, Monack D, Falkow S, Gleeson C, Kubo T, Holden**  
603 **DW.** 1997. Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding  
604 components of the type III secretion apparatus of *Salmonella* Pathogenicity Island 2.  
605 *Mol Microbiol* **24**:155-167.
- 606 24. **Beuzon CR, Holden DW.** 2001. Use of mixed infections with *Salmonella* strains to  
607 study virulence genes and their interactions in vivo. *Microbes Infect* **3**:1345-1352.
- 608 25. **Fields PI, Swanson RV, Haidaris CG, Heffron F.** 1986. Mutants of *Salmonella*  
609 typhimurium that cannot survive within the macrophage are avirulent. *Proc Natl Acad*  
610 *Sci U S A* **83**:5189-5193.

- 611 26. **Claudi B, Sprote P, Chirkova A, Personnic N, Zankl J, Schurmann N, Schmidt A,**  
612 **Bumann D.** 2014. Phenotypic variation of Salmonella in host tissues delays eradication  
613 by antimicrobial chemotherapy. *Cell* **158**:722-733.
- 614 27. **Ferla MP, Patrick WM.** 2014. Bacterial methionine biosynthesis. *Microbiology*  
615 **160**:1571-1584.
- 616 28. **Jeter RM, Olivera BM, Roth JR.** 1984. Salmonella typhimurium synthesizes cobalamin  
617 (vitamin B12) de novo under anaerobic growth conditions. *J Bacteriol* **159**:206-213.
- 618 29. **Bazurto JV, Farley KR, Downs DM.** 2016. An unexpected route to an essential  
619 cofactor: Escherichia coli relies on threonine for thiamine biosynthesis. *MBio* **7**:pii:  
620 e01840-15. doi: 10.1128/mBio.01840-15.
- 621 30. **Kim J, Copley SD.** 2007. Why metabolic enzymes are essential or nonessential for  
622 growth of Escherichia coli K12 on glucose. *Biochemistry* **46**:12501-12511.
- 623 31. **Morine MJ, Gu H, Myers RA, Bielawski JP.** 2009. Trade-offs between efficiency and  
624 robustness in bacterial metabolic networks are associated with niche breadth. *J Mol*  
625 *Evol* **68**:506-515.
- 626 32. **Jiang D, Zhou S, Chen YP.** 2009. Compensatory ability to null mutation in metabolic  
627 networks. *Biotechnol Bioeng* **103**:361-369.
- 628 33. **Brown RF, Stocker BA.** 1987. Salmonella typhi 205aTy, a strain with two attenuating  
629 auxotrophic characters, for use in laboratory teaching. *Infect Immun* **55**:892-898.
- 630 34. **O'Callaghan D, Maskell D, Liew FY, Easmon CS, Dougan G.** 1988. Characterization of  
631 aromatic- and purine-dependent Salmonella typhimurium: attention, persistence, and  
632 ability to induce protective immunity in BALB/c mice. *Infect Immun* **56**:419-423.

- 633 35. **Jackson M, Phalen SW, Lagranderie M, Ensergueix D, Chavarot P, Marchal G,**  
634 **McMurray DN, Gicquel B, Guilhot C.** 1999. Persistence and protective efficacy of a  
635 *Mycobacterium tuberculosis* auxotroph vaccine. *Infect Immun* **67**:2867-2873.
- 636 36. **Crawford RM, Van De Verg L, Yuan L, Hadfield TL, Warren RL, Drazek ES, Houg**  
637 **HH, Hammack C, Sasala K, Polsinelli T, Thompson J, Hoover DL.** 1996. Deletion of  
638 *purE* attenuates *Brucella melitensis* infection in mice. *Infect Immun* **64**:2188-2192.
- 639 37. **Pechous R, Celli J, Penoske R, Hayes SF, Frank DW, Zahrt TC.** 2006. Construction  
640 and characterization of an attenuated purine auxotroph in a *Francisella tularensis* live  
641 vaccine strain. *Infect Immun* **74**:4452-4461.
- 642 38. **Leung KY, Finlay BB.** 1991. Intracellular replication is essential for the virulence of  
643 *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **88**:11470-11474.
- 644 39. **Grant AJ, Morgan FJ, McKinley TJ, Foster GL, Maskell DJ, Mastroeni P.** 2012.  
645 Attenuated *Salmonella Typhimurium* lacking the pathogenicity island-2 type 3  
646 secretion system grow to high bacterial numbers inside phagocytes in mice. *PLoS*  
647 *Pathog* **8**:e1003070.
- 648 40. **Mantena RK, Wijburg OL, Vindurampulle C, Bennett-Wood VR, Walduck A,**  
649 **Drummond GR, Davies JK, Robins-Browne RM, Strugnell RA.** 2008. Reactive oxygen  
650 species are the major antibacterials against *Salmonella Typhimurium* purine  
651 auxotrophs in the phagosome of RAW 264.7 cells. *Cell Microbiol* **10**:1058-1073.
- 652 41. **Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JC.** 2003. Unravelling the  
653 biology of macrophage infection by gene expression profiling of intracellular  
654 *Salmonella enterica*. *Mol Microbiol* **47**:103-118.

- 655 42. **Pratt ZL, Chen B, Czuprynski CJ, Wong AC, Kaspar CW.** 2012. Characterization of  
656 osmotically induced filaments of *Salmonella enterica*. *Appl Environ Microbiol.* 2012;  
657 **78**:6704-13. doi: 10.1128/AEM.01784-12.
- 658 43. **Knudsen GM, Nielsen MB, Thomsen LE, Aabo S, Rychlik I, Olsen JE.** 2014. The role  
659 of ClpP, RpoS and CsrA in growth and filament formation of *Salmonella enterica*  
660 serovar Typhimurium at low temperature. *BMC Microbiol.* 2014; **14**:208. doi:  
661 10.1186/s12866-014-0208-4.
- 662 44. **Ejim LJ, D'Costa VM, Elowe NH, Loredó-Osti JC, Malo D, Wright GD.** 2004.  
663 Cystathionine beta-lyase is important for virulence of *Salmonella enterica* serovar  
664 Typhimurium. *Infect Immun* **72**:3310-3314.
- 665 45. **Lestrade P, Delrue RM, Danese I, Didembourg C, Taminiau B, Mertens P, De Bolle**  
666 **X, Tibor A, Tang CM, Letesson JJ.** 2000. Identification and characterization of in vivo  
667 attenuated mutants of *Brucella melitensis*. *Mol Microbiol* **38**:543-551.
- 668 46. **Basavanna S, Chimalapati S, Maqbool A, Rubbo B, Yuste J, Wilson RJ, Hosie A,**  
669 **Ogunniyi AD, Paton JC, Thomas G, Brown JS.** 2013. The effects of methionine  
670 acquisition and synthesis on *Streptococcus pneumoniae* growth and virulence. *PLoS*  
671 *One* **8**:e49638.
- 672 47. **Watson PR, Galyov EE, Paulin SM, Jones PW, Wallis TS.** 1998. Mutation of *invH*, but  
673 not *stn*, reduces *Salmonella*-induced enteritis in cattle. *Infect Immun.* **66**:1432-38.
- 674 48. **Chang AC, Cohen SN.** 1978. Construction and characterization of amplifiable  
675 multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol*  
676 **134**:1141-1156.  
677  
678

679 Table 1. Strains and plasmids used in the study.

680

Strain number	Strain	Genotype and marker <sup>#</sup>	Source
Jeo3774	<i>S. Typhimurium</i> 4/74	Wild- type	(47)
Jeo1473	<i>S. Typhimurium</i> $\Delta purM::Cm$	4/47 $\Delta STM474\_2603, cm^r$	This study
Jeo1516	<i>S. Typhimurium</i> $\Delta purM::Cm$ -comp.	4/47 $\Delta STM474\_2603+$ $STM474\_2603^{comp},$ $cm^r, amp^r$	This study
Jeo1496	<i>S. Typhimurium</i> $\Delta purT::Kan$	4/47 $\Delta STM474\_1915,$ $kan^r$	This study
Jeo1517	<i>S. Typhimurium</i> $\Delta purT::Kan$ -comp.	4/47 $\Delta STM474\_1915+$ $STM474\_1915^{comp},$ $kan^r, amp^r$	This study
Jeo1509	<i>S. Typhimurium</i> $\Delta purM::Cm/ \Delta purT::Kan$	4/74 $\Delta STM474\_2603/$ $\Delta STM474\_1915,$ $kan^r, cm^r$	This study
Jeo1512	<i>S. Typhimurium</i> $\Delta glyA::Kan$	4/47 $\Delta STM474\_2659,$ $kan^r$	(8)
Jeo1528	<i>S. Typhimurium</i>	4/47	This study

	$\Delta glyA::Cm$	$\Delta STM474\_2659$ , $cm^r$	
Jeo1570	<i>S. Typhimurium</i>	4/47 $\Delta STM474\_2659$	This study
	$\Delta glyA::Kan$ - comp.	+ $STM474\_2659^{comp}$ , $kan^r$ , $amp^r$	
Jeo1526	<i>S. Typhimurium</i>	4/47	This study
	$\Delta serA::Kan$	$\Delta STM474\_3209$ , $kan^r$	
Jeo1514	<i>S. Typhimurium</i>	4/47 $\Delta STM474\_3209$	This study
	$\Delta serA::Kan/ \Delta glyA::Cm$	+ $\Delta STM474\_2659$ $kan^r$ , $cm^r$	
Jeo1527	<i>S. Typhimurium</i>	4/74	This study
	$\Delta purT::kan/ \Delta glyA::cm$	$\Delta STM474\_1915/$ $\Delta STM474\_2659$ , $kan^r$ , $cm^r$	
Jeo1522	<i>S. Typhimurium</i>	4/74	This study
	$\Delta purT::kan/ \Delta serA::cam$	$\Delta STM474\_1915/$ $\Delta STM474\_3209$ , $kan^r$ , $cm^r$	
Jeo1521	<i>S. Typhimurium</i>	4/74	This study
	$\Delta purM::Kan/ \Delta glyA::Cm$	$\Delta STM474\_2603/$ $\Delta STM474\_2659$ , $kan^r$ , $cm^r$	
Jeo1525	<i>S. Typhimurium</i>	4/74	This study
	$\Delta purM::Kan/ \Delta serA::Cm$	$\Delta STM474\_2603/$ $\Delta STM474\_3209$ ,	

		kan <sup>r</sup> , cm <sup>r</sup>	
Jeo1523	<i>S. Typhimurium</i>	4/47	This study
	$\Delta purU::Kan$	$\Delta STM474\_1773,$	
		kan <sup>r</sup>	
Jeo1577	<i>S. Typhimurium</i> $\Delta purU -$	4/47	This study
	compl	$\Delta STM474\_1773+$	
		$\Delta STM474\_1773^{compl},$	
		amp <sup>r</sup>	
Jeo1572	<i>S. Typhimurium</i> $\Delta purU$	4/47	This study
	$/\Delta purT::kan$	$\Delta STM474\_1773,$	
		$\Delta STM474\_1915,$	
		kan <sup>r</sup>	
Jeo1529	<i>S. Typhimurium</i>	4/47	This study
	$\Delta gcvT::Cm$	$\Delta STM474\_3202, cm^r$	
Jeo1531	<i>S. Typhimurium</i>	4/74	This study
	$\Delta serA::Kan/ \Delta gcvT::Cm$	$\Delta STM474\_3209/$	
		$\Delta STM474\_3202,$	
		kan <sup>r</sup> , cm <sup>r</sup>	
Jeo1530	<i>S. Typhimurium</i>	4/74	This study
	$\Delta glyA::Kan/ \Delta gcvT::Cm$	$\Delta STM474\_2659/$	
		$\Delta STM474\_3202,$	
		kan <sup>r</sup> , cm <sup>r</sup>	
Jeo1574	<i>S. Typhimurium</i>	4/47	This study
	$\Delta metE::Kan$	$\Delta STM474\_4143,$	
		kan <sup>r</sup>	



Jeo1590	<i>S. Typhimurium</i>	4/47	This study
	$\Delta metH::Cm$	$\Delta STM474\_4378, Cm^r$	
Jeo1593	<i>S. Typhimurium</i>	4/74	This study
	$\Delta metE::Kan/$	$\Delta STM474\_4143/$	
	$\Delta metH::Cm$	$\Delta STM474\_4378,$ $kan^r, cm^r$	
Jeo1599	<i>S. Typhimurium</i>	4/74	This study
	$\Delta metE::Kan/$	$\Delta STM474\_4143/$	
	$\Delta metH::Cm-comp.$	$\Delta STM474\_4378/+$ $STM474\_4143^{comp}$	
Jeo1594	<i>S. Typhimurium</i>	4/74	This study
	$\Delta metE::Kan/$	$\Delta STM474\_4143/$	
	$\Delta metH::Cm,$	$\Delta STM474\_4378/$	
	$\Delta purT::Kan$	$\Delta STM474\_1915,$ $kan^r, cm^r$	
-	<i>S. Typhimurium</i>	4/47	(21)
	$\Delta ssaV::Kan$	$\Delta STM474\_1420,$ $kan^r$	
-	pKD3	Template plasmid for amplification of chloramphenicol resistance gene cassette. $Amp^r, Cm^r$	(20)
-	pKD4	Template plasmid for amplification of	(20)

		kanamycin	
		resistance gene	
		cassette. Amp <sup>r</sup> , Kan <sup>r</sup>	
-	pKD46	Lambda red	(20)
		mediated	
		recombination	
		system. Amp <sup>r</sup>	
-	pACYC177	Low copy number	(48)
		plasmid used for	
		complementation.	
		Amp <sup>r</sup> .	

---

681 \*Gene numbers refer to the 4/74 genome annotation in BioCyC ([www.biocyc.org](http://www.biocyc.org)). Cm<sup>r</sup>:  
682 chloramphenicol resistant through insertion of the chloramphenicol gene cassette from the plasmid  
683 pKD3. Kan<sup>r</sup>: Kanamycin resistant through the insertion of the kanamycin resistance gene cassette  
684 from the plasmid pKD4. Comp: Complemented *in trans* by cloning of the deleted gene on the  
685 plasmid pACY177.  
686

687 Table 2. Growth and Virulence of *S. Typhimurium* 4/74 *pur*-mutants.

Strain	Mutant	Growth rate <sup>a,b</sup> ( $\mu$ , hours <sup>-1</sup> )	Mice virulence (2) <sup>b</sup>	J774A.1 macrophage <sup>c</sup> (multiplication/20 h) <sup>b</sup>
Jeo3774	WT 4/74	0.37±0.017	1.00	9.7 ± 2.5
Jeo1473	$\Delta purN$	0.35±0.01	0.03 ± 0.0***	4.4 ± 3.5*
Jeo1516	$\Delta purN$ / pACYC177 <i>purN</i>	0.31±0.08	0.60 ± 0.2	ND
Jeo1496	$\Delta purT$	0.36±0.02	0.02 0.01***	8.0 ± 5.6
Jeo1517	$\Delta purT$ / pACYC177 <i>purT</i>	0.37±0.01	0.90 ± 0.3	ND
Jeo1509	$\Delta purN$ / $\Delta purT$	NG	0.00 ± 0.0 <sup>NT</sup>	0.3 ± 0.2****
Jeo1523	$\Delta purU$	0.25±0.002***	0.60 ± 0.8	4.6 ± 2.5*
Jeo1575	$\Delta purU$ / pACYC177 <i>purU</i>	0.15±0.002***	ND	2.1 ± 0.1*
Jeo1572	$\Delta purU$ / $\Delta purT$	0.38±0.01	1.63 ± 0.6**	ND

688 ND: not determined; NG: no growth in M9 + glucose medium.

689 a: Specific growth rate *in vitro* in M9+glucose (ln(2)/doubling time).

690 b: \*: p<0.05; \*\*: P-value <0.01 \*\*\*: p-value <0.001; \*\*\*\*: p<0.0001; NT: Cannot be tested as  
691 no mutant colonies were recovered from challenged mice.

692 c: Control 4/74  $\Delta ssaV$ : 2.1 ± 0.6\*\*\*

693

694 Table 3. Growth and virulence phenotypes of mutants in glycine and serine metabolism  
695 in *S. Typhimurium* 4/74.

Strain	Genotype	Growth rate <sup>a,b</sup> ( $\mu$ , hours <sup>-1</sup> )	Mice virulence (2)	Macrophage multiplication (1h to 21 h) <sup>c</sup>
Jeo3774	Wildtype (WT) 4/74	0.37 $\pm$ 0.017	1.00	6.9 $\pm$ 3.4
Jeo1529	$\Delta gcvT$	0.36 $\pm$ 0.002	0.03 $\pm$ 0.03 <sup>***e</sup>	7.2 $\pm$ 3.9
Jeo1531	$\Delta serA \Delta gcvT$	ND	0.05 $\pm$ 0.04 <sup>***</sup>	ND
Jeo1522	$\Delta serA \Delta purT$	NG	0.15 $\pm$ 0.15 <sup>***</sup>	ND
Jeo1512	$\Delta glyA$	0.03 $\pm$ 0.003	0.3 $\pm$ 0.4 <sup>*</sup>	0.3 $\pm$ 0.03 <sup>**</sup>
Jeo1512	$\Delta glyA$ +gly <sup>d</sup>	0.20 $\pm$ 0.01	NR	NR
Jeo1570	$\Delta glyA$ +pACYC177 <i>glyA</i>	0.35 $\pm$ 0.01	ND	5.6 $\pm$ 1.50 <sup>e</sup>
Jeo1514	$\Delta glyA \Delta serA$	NG	0.01 $\pm$ 0.01 <sup>***</sup>	ND
Jeo1530	$\Delta glyA \Delta gcvT$	NG	0.0 $\pm$ 0.0 <sup>NT(*)</sup>	ND
Jeo1521	$\Delta glyA \Delta purN$	0.03 $\pm$ 0.003	0.02 $\pm$ 0.02 <sup>***</sup>	1.2 $\pm$ 1.0 <sup>***</sup>

696 ND: not done; NG: no growth in M9 + glucose medium.

697 a: Specific growth rate *in vitro* in M9+glucose (ln(2)/doubling time).

698 b: \*: p<0.05; \*\*: P-value <0.01 \*\*\*: p-value <0.001; \*\*\*\*: p<0.0001;

699 NT: This strain could not be tested statistically as no mutant colonies were recovered from  
700 challenged mice. If for statistical purposes, one assumed one colony to be obtained from each  
701 mouse of this double mutant, the results would not have been significantly different from the  
702 competitive index of the  $\Delta gcvT$  mutant on its own (p=0.06) but significantly different from the  
703 *glyA* mutant (p=0.03).

704 c: Control 4/74  $\Delta ssaV$ : 2.1  $\pm$  0.6<sup>\*\*\*</sup>

705 d: Growth in M9 minimal medium with supplement of glycine (gly).

706 e: Multiplication rate is significantly different from Jeo1512 ( $\Delta g/yA$ ) and not different from Jeo3774  
707 (wild type strain).  
708

709 Table 4. Virulence phenotypes of *metE* and *metH* mutants in *S. Typhimurium* 4/74.

Strain number	Genotype	Growth rate <sup>a,b</sup> (μ) gene/h	Competitive index (2)	Multiplication rate in macrophages (1h to 21 h) <sup>d</sup>
Jeo3774	Wildtype (WT) 4/74 <sup>c</sup>	0.37±0.02	1.00	6.9 ± 3.4
Jeo1590	<i>ΔmetH</i>	0.20±0.01	0.4 ± 0.2	ND
Jeo1590	<i>ΔmetH+met<sup>c</sup></i>	0.33±0.03	NR	NR
Jeo1574	<i>ΔmetE</i>	0.06±0.01	0.0 ± 0.0	ND
Jeo1574	<i>ΔmetE+met<sup>c</sup></i>	0.23±0.05	NR	NR
Jeo1593	<i>ΔmetH ΔmetE</i>	NG	0.0 ± 0.0 <sup>NT</sup>	2.9 ± 0,4
Jeo1593	<i>ΔmetH ΔmetE+met<sup>c</sup></i>	0.26±0.005	NR	NR
Jeo1599	<i>ΔmetH ΔmetE</i> /pAYCY177 <i>metE</i>	0.24±0.002	0.00 ± 0.0 <sup>NT</sup>	ND

710 ND: not done; NG: no growth in M9 + glucose medium.

711 a: growth rate *in vitro* in M9+glucose.

712 b: \*: p<0.05; \*\*: P-value <0.01 \*\*\*: p-value <0.001; \*\*\*\*: p<0.0001; NT: Cannot be tested as  
713 no mutant colonies were recovered from challenged mice.

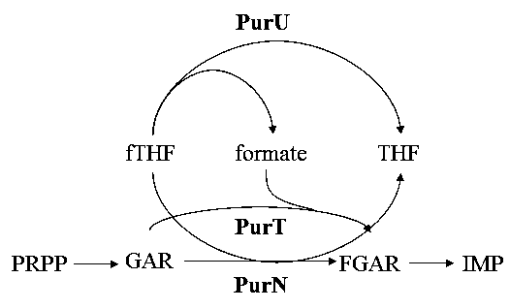
714 c: Growth in M9 minimal medium with supplement of methionine

715 d: Control 4/74 *ΔssaV*: 2.1 ± 0.6\*\*\*

716

717 Fig 1. The third reaction-step in the purine *de novo* synthesis carried out by the *in vitro* redundant  
718 enzymes PurN and PurT (Fig. 1A), and the interconnection between purine synthesis and the  
719 carbon-1 metabolism, where essential carbon-1 (C-1) units are produced (Fig 1B).  
720 (Fig 1A): PurN converts Gar to fGar using fTHF as formyl donor, while PurT converts Gar to fGar  
721 using formate as formyl donor. Formate is produced from fTHF by the enzyme PurU. (B): The  
722 production of C-1 units for amino acid and purine synthesis in *S. Typhimurium* happens when GlyA  
723 converts serine into glycine and when glycine is converted into ammonia and carbon dioxide by  
724 the glycine cleavage system. The pools of formyl-THF (fTHF), THF and methylene-THF (mTHF) are  
725 shared between the purine and the Carbon-1 synthesis pathways.

**Fig. 1A**



**Fig. 1B**

